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Dual Function of p38α MAPK in Colon Cancer: Suppression of Colitis-Associated Tumor Initiation but Requirement for Cancer Cell Survival

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SUMMARY

Colorectal cancer is frequently associated with chronic inflammation, with the intestinal epithelial barrier playing an important protective role against the infections and injuries that cause colitis. The p38α pathway regulates inflammatory responses but can also suppress tumor initiation in epithelial cells. We have found that p38α signaling has a dual function in colorectal tumorigenesis. On one side, p38α protects intestinal epithelial cells against colitis-associated colon cancer by regulating intestinal epithelial barrier function. Accordingly, p38α downregulation results in enhanced colitis-induced epithelial damage and inflammation, which potentiates colon tumor formation. Surprisingly, inhibition of p38α in transformed colon epithelial cells reduces tumor burden. Thus, p38α suppresses inflammation-associated epithelial damage and tumorigenesis but contributes to the proliferation and survival of tumor cells.

INTRODUCTION

Cancer is a complex disease that arises through a multistep, mutagenic process. Acquisition of cancer cell features involves changes in the wiring of signaling pathways that are normally tightly regulated to control processes such as cell proliferation, survival, and differentiation, which are critical to maintain tissue homeostasis.

Colorectal tumors are of epithelial origin and develop from sequential mutations in the Wnt signaling pathway, K-Ras, p53, and the transforming growth factor (TGF)-β pathway (Fearon and Vogelstein, 1990; Ullman and Itzkowitz, 2011). Most of these mutations are induced by environmental factors, such as chronic inflammation of the colon epithelia. It is well established that chronic inflammation may lead to increased risk of several types of cancer (Mantovani et al., 2008; Schetter et al., 2010). For example, patients with inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis, have higher risk of colorectal cancer development than the healthy population (Gillen et al., 1994). The intestinal epithelial barrier plays an important role protecting the gastrointestinal tract. This barrier consists of a mucus layer, which acts as a physical barrier, and the epithelial layer firmly adhered with epithelial tight junctions (Rescigno, 2011). Defects in the barrier function allow direct contact of intestinal mucosa with the luminal invading pathogens and ingested toxins promoting inflammatory responses. IBD and colon cancer have been associated with abnormal epithelial barrier function (Schmitz et al., 1999; Westbrook et al., 2010; Grivennikov et al., 2012). Moreover, mouse models with defects in epithelial barrier have increased susceptibility to colitis and colorectal tumor formation, providing evidence for the regulation of inflammation and tumorigenesis

Significance

Inflammatory bowel disease (IBD) patients have higher risk of colorectal cancer development, but the knowledge of signaling pathways involved in the pathogenesis of IBD is limited. In this study, we show that p38α signaling in epithelial cells is required to maintain epithelial barrier function and intestinal homeostasis, controlling the severity of colitis. Thus, p38α negatively regulates the formation of colitis-associated colon tumors. It is striking that transformed intestinal epithelial cells rely on p38α signaling for survival and proliferation. The reduced colon tumor burden observed on genetic downregulation or pharmacological inhibition of p38α suggests therapeutic opportunities for colorectal cancer by targeting this pathway.

by the intestinal epithelial barrier in vivo (Laukoetter et al., 2007; Van der Sluis et al., 2006).

p38α is a mitogen-activated protein kinase (MAPK) that regulates the cellular responses to stress but also has other functions, including crucial roles in inflammation and tissue homeostasis (Cuenda and Rousseau, 2007). Genetic inactivation of p38α in myeloid cells supports the importance of this pathway in cytokine production and inflammatory responses (Kang et al., 2008; Kim et al., 2008a). There is also evidence that p38α signaling controls tissue homeostasis by inducing differentiation while negatively regulating proliferation of many cell types, including epithelial cells (Cuadrado and Nebreda, 2010). It is interesting that p38α-deficient mice are more susceptible to K-Ras-induced lung tumorigenesis and to diethylnitrosamine (DEN)/phenobarbital (Pb)-induced liver cancer, indicating that p38α can function as a tumor suppressor in vivo (Hui et al., 2007; Ventura et al., 2007). However, there is little evidence for p38α inactivating mutations in tumors, which probably reflects that cancer cells take advantage of the ability of this signaling pathway to control multiple cellular processes. In line with this idea, chemical inhibitors of p38α have been shown to impair the proliferation of some human cancer cell lines (Wagner and Nebreda, 2009).

Given the role of the p38α pathway in orchestrating inflammatory responses while negatively regulating epithelial cell transformation, we investigated how these two functions are balanced in colon epithelia during colitis-associated colorectal cancer (CAC).

RESULTS

Downregulation of p38α in Intestinal Epithelial Cells Increases CAC

We generated mice expressing Villin-Cre and p38α-lox alleles to downregulate p38α in intestinal epithelial cells (IEC) (p38α-Δ^{IEC}). The efficiency of p38α downregulation was confirmed by western blotting in isolated IEC and in whole-colon lysates (Figure 1A). To investigate the role of p38α in CAC, we used a protocol that combines the carcinogen azoxymethane (AOM) with dextran sodium sulfate (DSS)-induced colitis (Figure 1B). When we applied the AOM/DSS protocol, p38α-Δ^{IEC} mice and wild-type (WT) littermates developed colon tumors mainly in the distal to middle colon (Figure 1C), which is the predominant localization of human colorectal tumors. We noticed that p38α-Δ^{IEC} mice had more macroscopic tumors (Figure 1D; Figure S1A available online), and the average tumor load was also higher but the average size was not affected (Figure 1D). Histological analyses confirmed more low-grade and high-grade tumors in p38α-Δ^{IEC} mice, but the relative proportion of low- versus high-grade tumors was similar in p38α-Δ^{IEC} and WT mice (Figure 1E). We also analyzed cell proliferation and apoptosis in these tumors and found no significant differences between WT and p38α-Δ^{IEC} mice (Figures 1F and S1B).

Inflammatory mediators are upregulated during AOM/DSS-induced colon tumorigenesis. We found that AOM/DSS-treated p38α-Δ^{IEC} mice showed higher COX-2 and interleukin (IL)-6 mRNA levels in the colon than the treated WT mice (Figure S1C). It is interesting that circulating IL-6 levels were also higher in serum from p38α-Δ^{IEC} mice at the end of the AOM/DSS protocol (Figure S1D). This is consistent with the known importance of IL-6 in colon tumor development.

Analysis of genomic DNA by quantitative PCR (qPCR) showed a substantial deletion of the floxed exon 2 of p38α in EpCAM$^+$ tumor epithelial cells, but not in CD45$^+$ leukocytes, from p38α-Δ^{IEC} mice versus WT mice (Figure 1G). Western blot analysis confirmed the downregulation of p38α protein in colon tumors from p38α-Δ^{IEC} mice (Figure S1E). The increased tumor burden observed in p38α-Δ^{IEC} mice without apparent effect on tumor size suggests that epithelial p38α signaling suppresses CAC by regulating tumor initiation.

p38α-Δ^{IEC} Mice Are More Susceptible to DSS-Induced Colitis and Epithelial Damage

AOM is a potent DNA damage-inducing agent that induces rapid p53-dependent apoptosis of IEC at the crypt base (Toft et al., 1999). However, AOM-induced DNA damage and apoptosis were similar in the colon from WT and p38α-Δ^{IEC} mice (Figures S2A and S2B), suggesting that the differences in tumorigenesis between WT and p38α-Δ^{IEC} mice were not due to a different initial response of IEC to AOM.

Next, we investigated the role of p38α in DSS-induced acute colitis, which is critical for AOM/DSS-induced tumorigenesis. After 5 days of DSS administration, p38α-Δ^{IEC} mice lost more body weight than WT mice (Figure 2A) suggesting that p38α-Δ^{IEC} mice probably had enhanced inflammation and intestinal damage, as body weight loss is one of the indicators for the severity of DSS-induced colitis. To confirm this possibility, mice were given DSS for 5 days and analyzed 1 or 3 days later. At both days 6 and 9, the WT colon showed minimal to mild inflammation, while colon from p38α-Δ^{IEC} mice showed moderate to severe inflammation, with many areas of complete crypt loss and erosions (Figure 2B). We also noticed that DSS-treated p38α-Δ^{IEC} mice had significantly more epithelial damage in the distal-middle part of the colon (Figure 2C).

Increased Inflammatory Cell Infiltration in p38α-Δ^{IEC} Mice Treated with DSS

Since p38α-Δ^{IEC} mice showed higher epithelial damage and inflammation, we characterized immune cell infiltration in the colon from DSS-treated mice. At day 6, we found a higher number of infiltrating cells in the colon of p38α-Δ^{IEC} mice than in WT mice (Figure 2D). Flow cytometry analyses showed more leukocytes (CD45$^+$ cells) in both epithelial/intraepithelial and lamina propria fractions of p38α-Δ^{IEC} mice. B cells (CD45$^+$CD19$^+$) also accumulated more in the lamina propria of p38α-Δ^{IEC} mice, whereas neutrophil (CD45$^+$CD11b$^+$Gr1$^+$) infiltration was very similar in the colons of WT and p38α-Δ^{IEC} mice (Figure S2C). We also found more macrophages in the colon of DSS-treated p38α-Δ^{IEC} mice compared to WT mice, whereas untreated mice showed no differences in macrophage infiltration (Figure S2D).

Infiltrating immune cells produce cytokines and chemokines to resolve the inflammation process. We found that IL-6 and COX-2 mRNAs were both upregulated in the colon from DSS-treated p38α-Δ^{IEC} mice compared to WT mice, whereas IL-1α and tumor necrosis factor α (TNF-α) mRNAs did not change. Basal expression levels of these mRNAs were comparable in untreated WT and p38α-Δ^{IEC} mice (Figure 2E). We also found higher IL-6

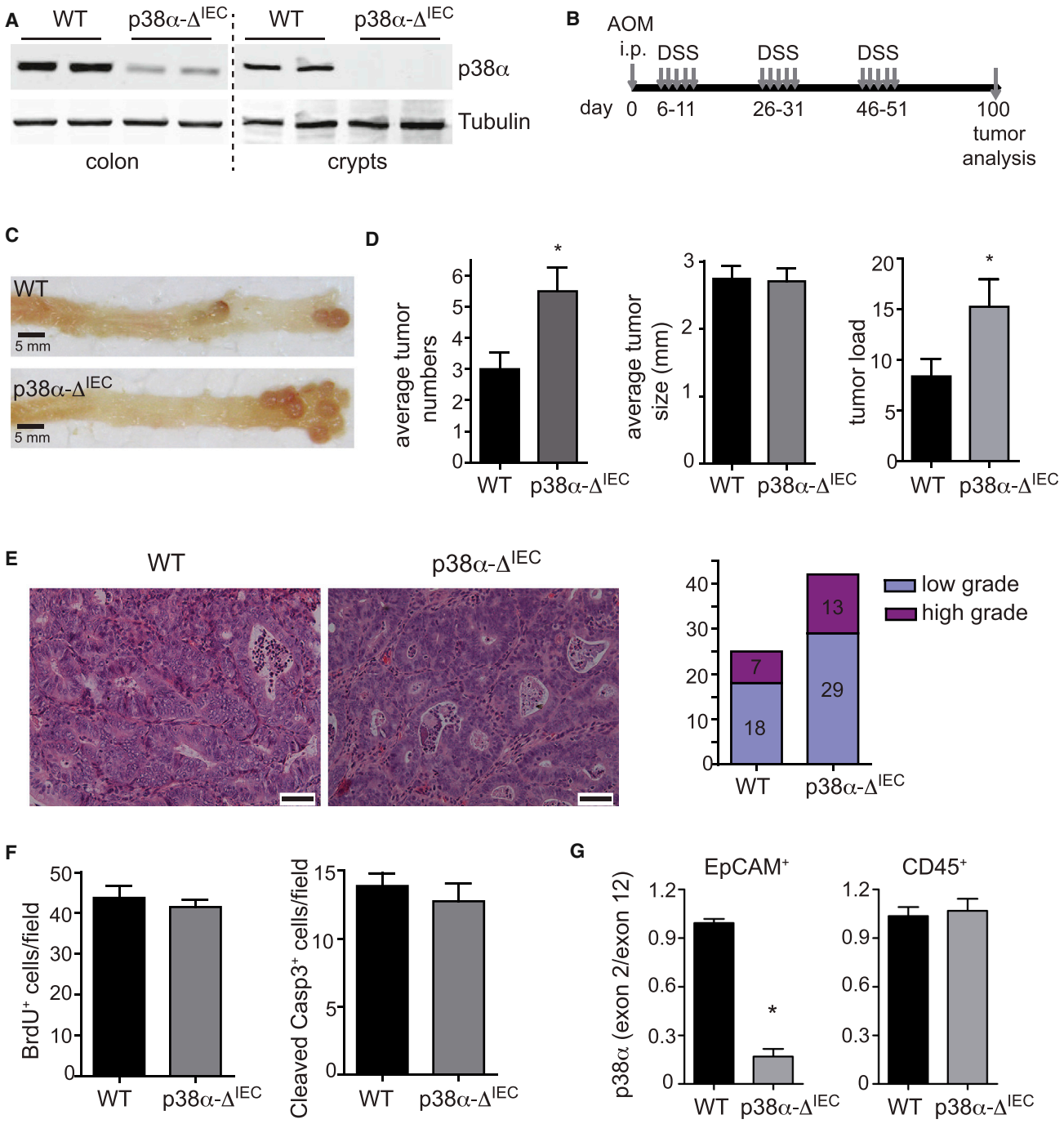


Figure 1. Downregulation of p38α in IEC Increases Susceptibility to AOM/DSS-Induced Colon Tumorigenesis

(A) Western blotting of p38α in whole-colon lysates (colon) and in isolated IEC (crypts).

(B) Schematic representation of the AOM/DSS protocol. i.p., intraperitoneal.

(C) Representative images of colon tumors.

(D) Average tumor number, size, and load. Data represent means ± SEM ($n \geq 6$). *$p < 0.05$.

(E) Representative images of H&E-stained colon tumors at the end of the AOM/DSS protocol. Scale bars, 50 μm. Tumors were microscopically analyzed and classified into low or high grade (n = 8).

(F) Colon sections were stained for bromodeoxyuridine (BrdU) or cleaved caspase 3 and quantified. Data represent means ± SEM (n = 4).

(G) Relative amount of the floxed exon 2 of p38α versus exon 12 (as a control) was determined by qPCR in EpCAM$^+$ epithelial cells and CD45$^+$ leukocytes. Data are means ± SEM ($n \geq 11$). *$p < 0.05$.

See also Figure S1.

protein levels both in colon lysates and in blood serum from DSS-treated p38α-Δ^{IEC} mice (Figure 2F). The increased IL-6 levels observed in p38α-Δ^{IEC} mice were unexpected, since the p38α pathway is known to positively regulate IL-6 expression in several cell types (Cuenda and Rousseau, 2007). Expression analysis using flow-cytometry-isolated cells showed that IL-6, IL-1α, and COX-2 mRNAs were all upregulated in IEC from DSS-treated p38α-Δ^{IEC} mice compared to WT mice, whereas TNF-α mRNA levels did not change. In contrast, the aforementioned inflammatory mediators were expressed at similar levels in the leukocytes from colon of WT or p38α-Δ^{IEC} mice (Figure S2E), which is consistent with the leukocytes being WT in both cases. Thus, the upregulation of IL-6 and COX-2 can be probably explained by both the presence of more infiltrating immune cells in colon of p38α-Δ^{IEC} mice and the enhanced expression of these cytokines in p38α-deficient IEC. Consistent with the increased IL-6 expression observed in DSS-treated p38α-Δ^{IEC} mice, we also detected phosphorylation and degradation of IκBα, suggesting activation of the NF-κB pathway, as well as enhanced phosphorylation of STAT3, a downstream target of IL-6 family receptors (Figure 2G).

Loss of p38α in IEC Induces Apoptosis and Hyperproliferation on DSS Treatment

Epithelial apoptosis is one of the mechanisms by which DSS can induce intestinal inflammation and colitis. We found that treatment with DSS induced more apoptosis in p38α-Δ^{IEC} mice than in WT mice (Figure 3A). Western blotting of IEC isolated from WT mice confirmed that DSS treatment increased the activating phosphorylation of p38α (Figure 3B). Analysis of Bcl-2 family members revealed that the proapoptotic protein Bak was significantly upregulated in DSS-treated p38α-Δ^{IEC} mice, whereas Bax and the antiapoptotic proteins Mcl-1, Bcl-2, and Bcl-XL were expressed at similar levels in DSS-treated WT and p38α-Δ^{IEC} mice (Figure 3B). In contrast, p53 expression and JNK phosphorylation levels were not changed (Figure 3B), suggesting that these pathways do not contribute to the enhanced apoptosis observed in DSS-treated p38α-Δ^{IEC} mice. Therefore, increased apoptosis, probably mediated by upregulation of Bak, could explain the increased epithelial damage and inflammation observed in the p38α-Δ^{IEC} mice on DSS treatment.

In response to the DSS-induced damage, the intestinal epithelium starts the repair and regeneration process by increasing cell proliferation. Since we detected more IL-6, an important regulator of the proliferation and survival of IEC (Bollrath et al., 2009; Grivennikov et al., 2009), we analyzed cell proliferation in the colon from DSS-treated mice. At day 6, we observed similar cell proliferation levels in intact crypts of WT and p38α-Δ^{IEC} mice; however, p38α-Δ^{IEC} mice had more proliferative cells than WT mice had in the areas of complete crypt loss, which probably represents a mixture of IEC and inflammatory cells (Figure 3C). At days 9 and 13, we found higher proliferation rates in IEC from WT and p38α-Δ^{IEC} mice compared to untreated mice. It is surprising that IEC proliferation was much higher in p38α-Δ^{IEC} than in WT mice during epithelium regeneration (Figure 3C). These data suggest that p38α downregulation results in IEC hyperproliferation after DSS-induced epithelial damage.

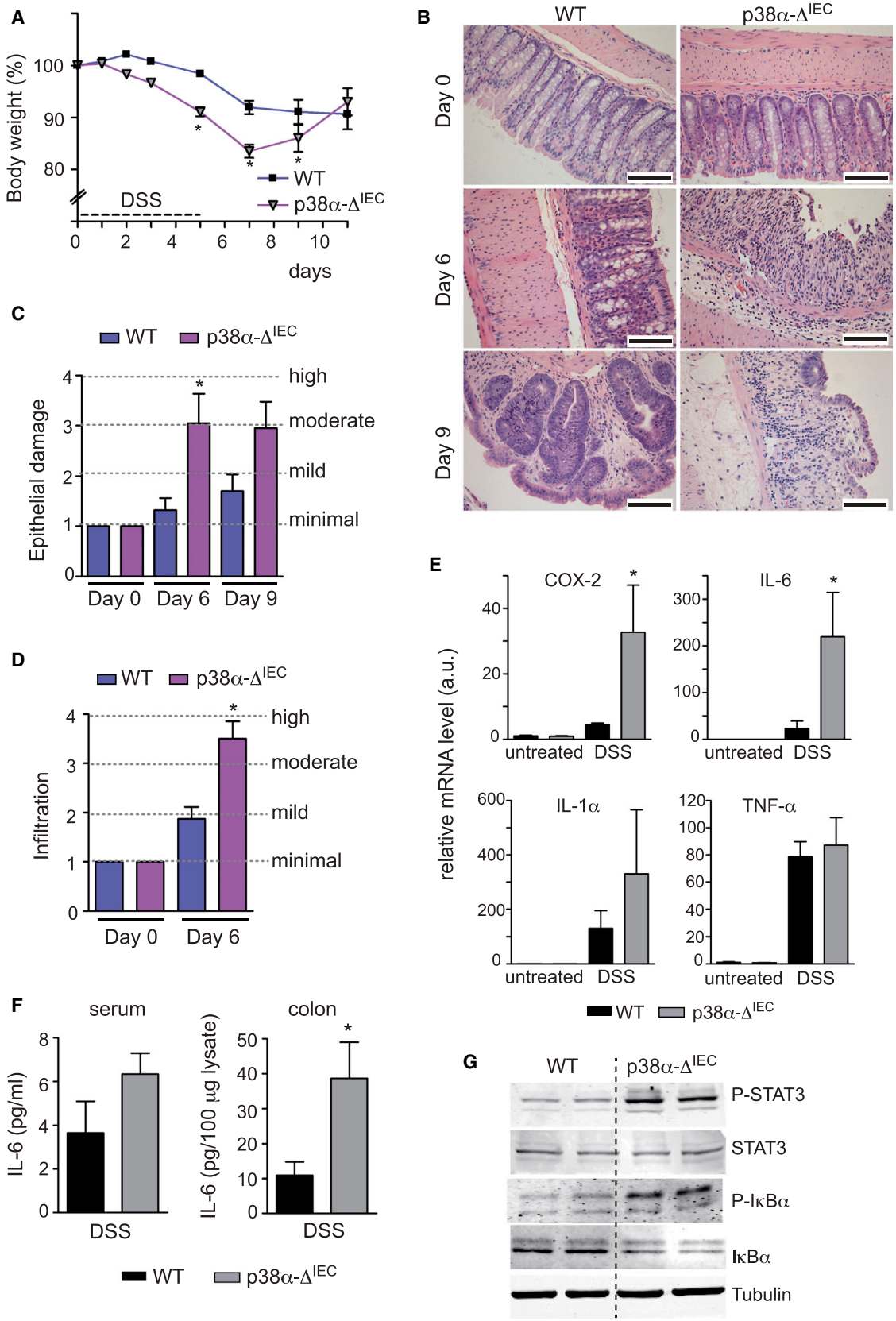
DSS Treatment Suffices to Induce Colon Tumors in p38α-Δ^{IEC} Mice

Unchecked compensatory proliferation and regeneration induced in response to repetitive tissue injury can promote tumorigenesis (Kuraishy et al., 2011). To test this possibility, we treated mice with three cycles of DSS but in the absence of the AOM carcinogen (Figure 4A). Macroscopic examination revealed that WT mice did not develop any tumors; however, about 60% of the p38α-Δ^{IEC} mice developed at least one colon tumor (Figure 4A). Histological examination confirmed the presence of colon tumors in p38α-Δ^{IEC} mice, which also contained more aberrant crypt foci with hyperchromatism of nuclei and hyperplastic crypts compared to WT mice (Figure 4B). The tumors formed in p38α-Δ^{IEC} mice had mutated β-catenin, corresponding to a GSK3β phosphorylation site, which leads to β-catenin activation and nuclear localization (Figures S3A and S3B). In addition, there were more macrophages in colon from p38α-Δ^{IEC} mice than from WT mice (Figure 4C). The inflammatory mediators COX-2, IL-6, TNF-α, and IL-12p40 were also expressed at higher levels in the colon from p38α-Δ^{IEC} mice than from WT mice (Figure 4D). Taking together, these data suggest that repeated DSS-induced epithelial injury in the absence of p38α results in uncontrolled hyperproliferation of IEC and a protumorigenic microenvironment that ultimately can induce colon hyperplasia and tumor formation.

p38α Regulates Homeostasis and Barrier Function in Normal Intestinal Epithelium

Alterations in epithelial barrier function have been associated with IBD and colon cancer (Grivennikov et al., 2012; Westbrook et al., 2010). We hypothesized that the increased epithelial damage and inflammation observed on p38α downregulation could be due to altered intestinal homeostasis and barrier function. In agreement with a recent report (Otsuka et al., 2010), we found that p38α downregulation in IEC had no effect on colon morphology, although p38α-Δ^{IEC} mice had more proliferative colon cells than the WT littermates. We also analyzed two main types of differentiated colon cells and found fewer chromogranin A^+ (ChgA) enteroendocrine cells and periodic acid-Schiff $^+$ (PAS) goblet cells in p38α-Δ^{IEC} mice compared to WT mice (Figure 5A; Figure S4A). Expression analysis confirmed upregulation of genes encoding proteins associated with cell proliferation (Ki67 and cyclin D1) and downregulation of differentiation-related genes (*MUC-2*, *TFF3*, and *ChgA*) in p38α-Δ^{IEC} mice (Figure S4B). These results indicate that p38α signaling can modulate both proliferation and differentiation of IEC.

Epithelial tight junctions are intercellular junctional complexes that regulate the paracellular permeability and are important for integrity of the epithelial barrier. Each tight junction contains a series of apparent fusions, called kissing points, where the intercellular space is completely obliterated (Tsukita et al., 2001). To investigate the epithelial barrier function, we first analyzed the effect of p38α downregulation on intestinal permeability. Using fluorescein isothiocyanate (FITC)-dextran, we found that in vivo intestinal permeability was notably increased in p38α-Δ^{IEC} mice (Figure 5B). Electron microscopy analysis revealed that epithelial tight junctions in colon from p38α-Δ^{IEC} mice were morphologically disrupted and had fewer kissing points compared to WT mice (Figures 5C and S4C). ZO-1 is a key



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component of tight junctions, which helps other proteins such as claudins and occludin to assemble at a tight junction (McNeil et al., 2006). Expression of ZO-1 was reduced in p38α-Δ^{IEC} mice, while claudin-1 and occludin seemed to be expressed at similar levels in WT and p38α-Δ^{IEC} mice (Figures 5D and S4D). These results suggest a role for p38α in the regulation of intestinal epithelia tight junction assembly and barrier function. In agreement with a recent study (Grivennikov et al., 2012), we also found upregulation of IL-23 mRNA in colon tumors versus normal tissue, but no differences were detected between WT and p38α-Δ^{IEC} mice. Similarly, IL-17 mRNA was expressed at similar levels in WT and p38α-Δ^{IEC} mice (Figure S4E). Thus, the epithelial barrier defect observed in p38α-Δ^{IEC} mice does not seem to be associated with IL-23 and IL-17 upregulation, but it is probably a consequence of the regulation by p38α of epithelial tight junction assembly.

To investigate whether intestinal barrier defects have a causal role in the enhanced epithelial damage induced by DSS in p38α-Δ^{IEC} mice, we used the probiotic mixture VSL#3 to protect the epithelial barrier (Mennigen et al., 2009). It is interesting that VSL#3 treatment for 7 days rescued the increased intestinal permeability observed in p38α-Δ^{IEC} mice (Figure 5E). Moreover, VSL#3 also rescued the enhanced apoptosis, the body weight loss, and the epithelial damage induced by DSS in p38α-Δ^{IEC} mice (Figures 5F–5H). These results support that the impaired epithelial barrier function contributes to the DSS-induced phenotypes of p38α-Δ^{IEC} mice, and it seems therefore likely that the epithelial barrier defect also contributes to the enhanced CAC observed in p38α-Δ^{IEC} mice.

Downregulation of p38α in Tumor Epithelial Cells Reduces Tumor Burden

Chemical inhibitors of p38α have been reported to inhibit proliferation in some cancer cells, including human colon cancer cell lines (Wagner and Nebreda, 2009). However, our genetic analysis indicated that p38α suppressed colon tumor formation. To clarify the role of p38α in colon tumorigenesis, we generated a mouse line with Villin-CreERT2 and p38α-lox alleles (p38α-$\Delta^{IEC\text{-}ERT2}$). We confirmed that treatment with 4-hydroxy tamoxifen (4-OHT) induced p38α downregulation in colon but not in other tissues of p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure S5A). Next, we verified our results using constitutive Villin-Cre by inducing p38α downregulation before DSS or AOM/DSS treatments (Figure S5B). We found that 4-OHT-treated p38α-$\Delta^{IEC\text{-}ERT2}$ mice developed more tumors than WT mice, but of similar size (Figure S5C). Moreover, after DSS treatment, p38α-$\Delta^{IEC\text{-}ERT2}$ mice lost more body weight than WT mice, as in the case of p38α-Δ^{IEC} mice (Figure S5D).

We next investigated the effect of p38α downregulation once colon tumors were formed. First, mice were subjected to the AOM/DSS protocol, and the presence of colon tumors was confirmed 65 days after the AOM injection. Then, mice with p38α-lox, and either with or without Villin-CreERT2, were injected with 4-OHT (Figure 6A). Three days after 4-OHT injection, p38α downregulation was poor, and no differences were detected in tumor number and size (Figure S5E). The downregulation of p38α improved at 10 days after 4-OHT injection when both tumor number and size started to decrease (Figure S5F). It is interesting that, 20 days after the last 4-OHT injection, tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice were mostly p38α deficient, as determined by western blotting (Figure 6B), by immunohistochemistry (IHC) (Figure S5G), and by analyzing the deletion of the floxed exon 2 of p38α in genomic DNA (Figures S5H and S5I). At this time, we observed that the average number, size, and load of macroscopic tumors were significantly reduced in p38α-$\Delta^{IEC\text{-}ERT2}$ mice compared with WT mice (Figure 6C and 6D). Of note, tumor burden and average tumor size both increased in WT mice compared to those of the initial tumors, suggesting that tumors continue to grow after 4-OHT injection (Figure 6D). When tumor size distribution was analyzed, we found fewer big colon tumors on p38α downregulation in tumor epithelial cells (Figure 6D). However, histological analysis revealed no differences in tumors from WT or p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure 6E). These results indicate a protumorigenic role for p38α in transformed colon epithelia cells.

Downregulation of p38α in Tumor Epithelial Cells Does Not Affect Tumor Permeability and Invasiveness

Recent studies described the importance of epithelial barrier function in colorectal tumors and invasiveness (Grivennikov et al., 2012; Schwitalla et al., 2013). We therefore investigated if the downregulation of p38α could result in more aggressive colon tumors. To test this hypothesis, p38α-$\Delta^{IEC\text{-}ERT2}$ mice were analyzed 45 days after 4-OHT injections. Initial experiments showed no differences in tumor burden or grading (Figures S6A and S6B), but western blotting revealed significant amounts of p38α in these tumors, probably corresponding to p38α-expressing escaper cells that repopulated the tumors in p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure S6C). To circumvent this problem, we performed two rounds of 4-OHT injections (Figure S6D). In this case, we confirmed the sustained downregulation of p38α in the tumors (Figure S6E), which correlated with reduced tumor number and size in p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure S6F). However, no differences were detected in histological tumor grading or aggressiveness between p38α-$\Delta^{IEC\text{-}ERT2}$ and WT mice (Figure S6G), suggesting that constant inhibition of p38α signaling is required

Figure 2. Downregulation of p38α in IEC Increases Susceptibility to DSS-Induced Colitis and Immune Cell Infiltration

(A) DSS was administered in drinking water for 5 days, and body weight was recorded. Data are means ± SEM ($n \geq 8$). *$p < 0.05$.

(B) Representative H&E-stained colon sections from mice either untreated or treated with DSS for 5 days and analyzed at days 6 and 9. Scale bars, 100 μm.

(C and D) Quantification of DSS-induced epithelial damage (C) and immune cell infiltration (D) in H&E-stained colon sections. Data represent means ± SEM ($n = 4$). *$p < 0.05$.

(E) Relative mRNA expression levels of inflammatory mediators in the distal colon of untreated and DSS-treated mice were determined by quantitative reverse transcription PCR (qRT-PCR). Expression levels in untreated WT and p38α-Δ^{IEC} mice were the same and were given the value of 1. Data are means ± SEM ($n = 4$). *$p < 0.05$.

(F) IL-6 protein levels in blood serum and whole-colon lysates of DSS-treated mice. Data are means ± SEM ($n = 5$).

(G) Colon lysates were prepared from DSS-treated mice and were analyzed by western blotting (one mouse per lane) with the indicated antibodies.

See also Figure S2.

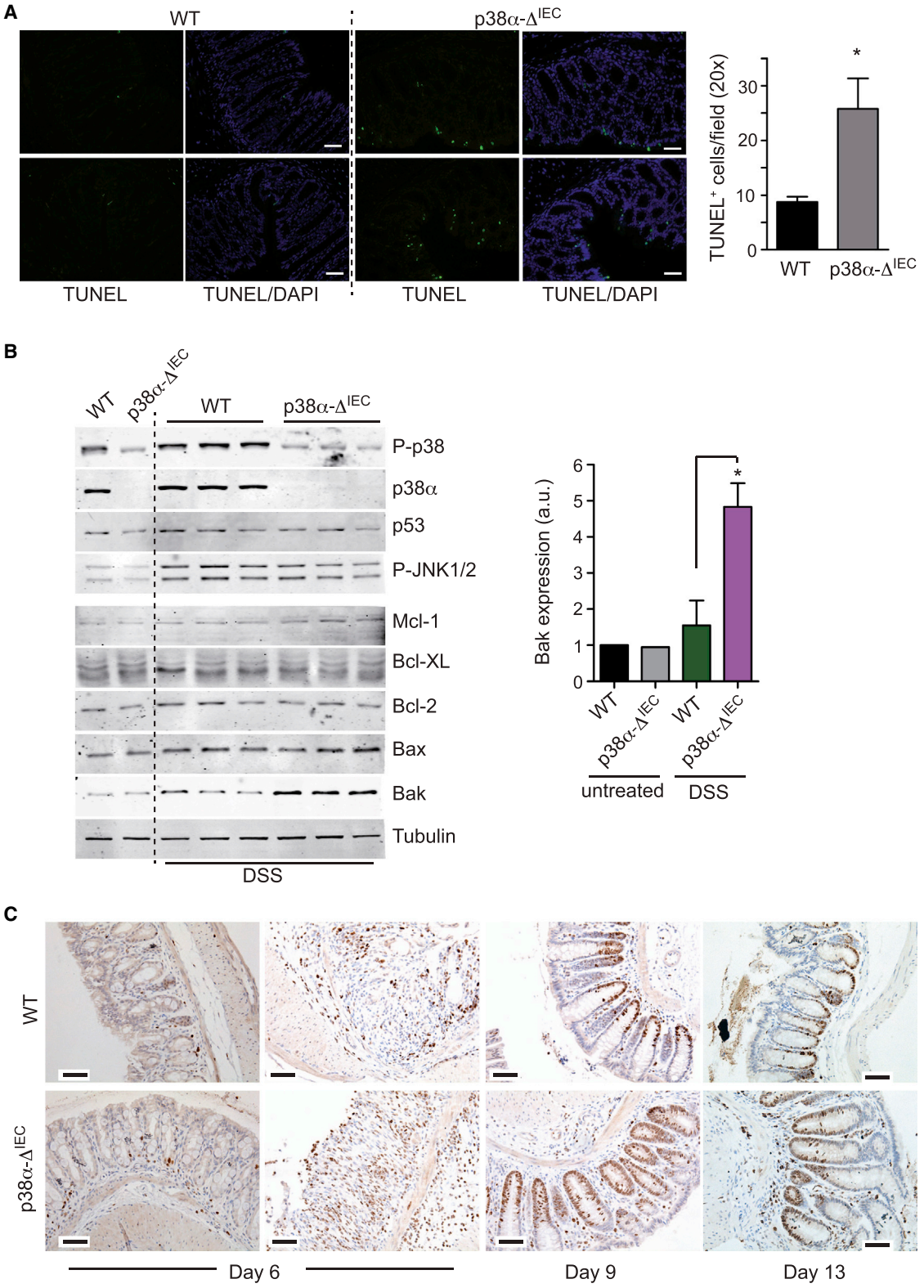

Figure 3. Downregulation of p38α in IEC Results in Enhanced DSS-Induced Apoptosis and Compensatory Proliferation

(A) Representative TUNEL staining of colon sections after 3 days of treatment with 3% DSS. Quantification is shown in the histogram. Data represent means ± SEM (n = 3). *p < 0.05.

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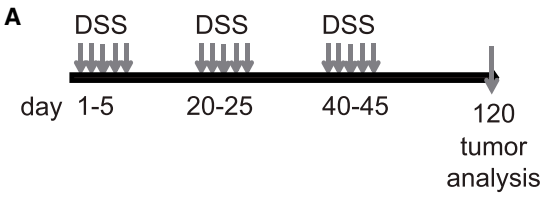
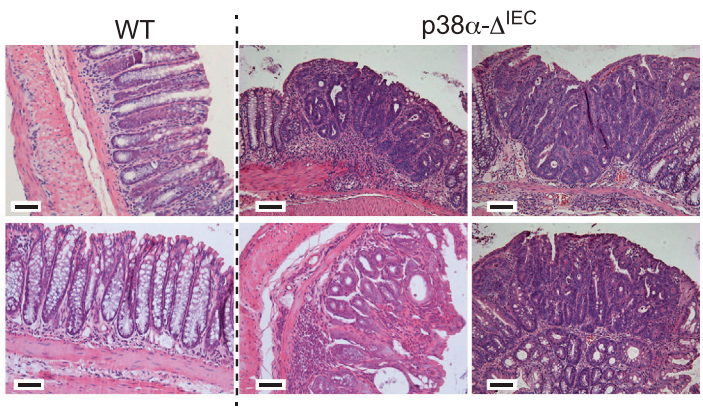
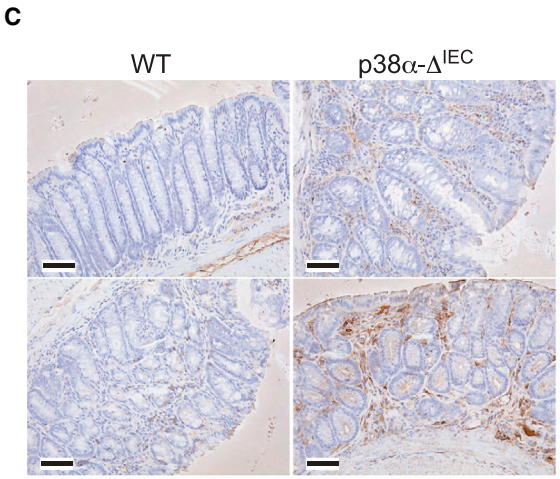


	WT	p38α-Δ^{IEC}
Number of mice	5	3 2 2
Tumors/mouse	0	0 1 2
Mice with tumors	0	57%





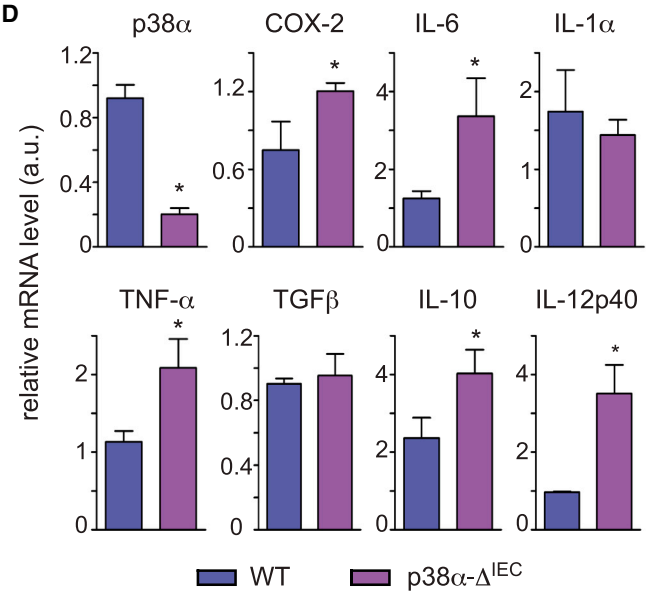


Figure 4. DSS Alone Suffices to Induce Colon Tumors in p38α-Δ^{IEC} Mice

(A) Schematic representation of the DSS protocol used to induce chronic colitis (top) and the summarized results (bottom).

(B) Representative images of H&E-stained colon sections at the end of the DSS treatment.

(C) Colon sections from mice treated with three cycles of DSS were stained for F4/80 to detect macrophages.

(D) Relative mRNA expression levels of the indicated genes in mouse colons were determined by qRT-PCR. Data are means ± SEM (n = 4). *p < 0.05.

Scale bars, 50 μm.

See also Figure S3.

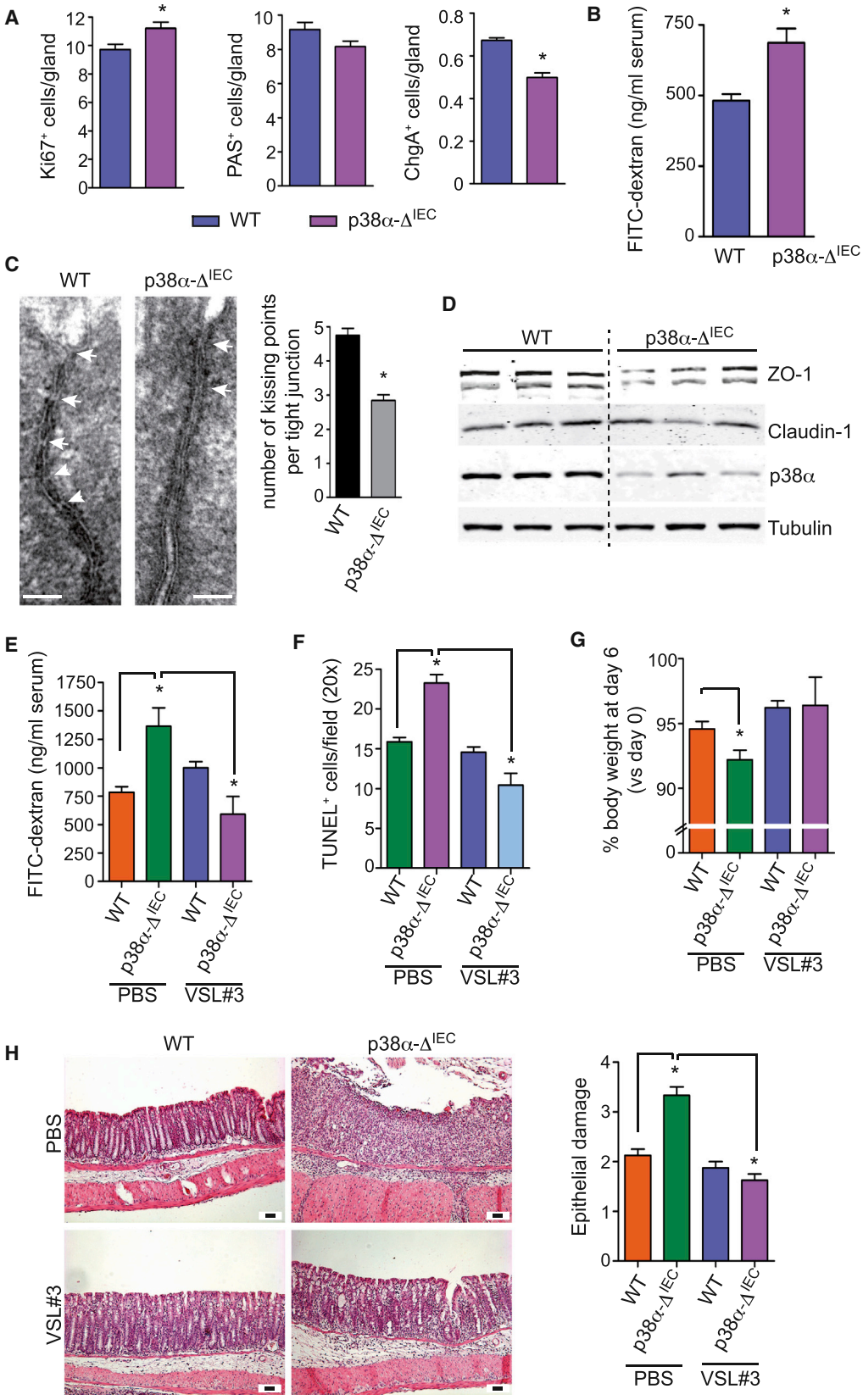
to reduce colon tumor burden but that this apparently has no effect on tumor aggressiveness.

Next, we measured intestinal permeability and found that, similar to p38α-Δ^{IEC} mice, permeability was higher in untreated p38α-Δ$^{IEC\text{-}ERT2}$ mice than in WT mice, but we could detect no differences in tumor permeability between WT and p38α-Δ$^{IEC\text{-}ERT2}$ mice (Figure S6H; see also Supplemental Experimental Procedures for tumor permeability calculation). In agreement with the permeability analysis, we found that p38α downregulation in colon tumors did not affect expression of the tight junction components ZO-1, ZO-2, Claudin-1, Occludin, and JAM-C (Figures S6I and S6J). Taken together, these results indicate that the reduced tumor burden observed in p38α-Δ$^{IEC\text{-}ERT2}$ mice is unlikely to be associated with enhanced tumor

(B) Colon epithelial cells were obtained from untreated or DSS-treated mice, and samples were analyzed by western blotting (one mouse per lane) with the indicated antibodies. The histogram shows the quantification of Bak expression. Data represent means ± SEM (n = 3). *p < 0.05.

(C) Sections from the distal colon of mice treated with 2% DSS for 5 days were stained for Ki67 at days 6, 9, and 13. For day 6: the left panels show an area with intact crypts; the right panels show an area with damage and crypt loss. Panels for days 9 and 13 show regenerating epithelia.

Scale bars, 50 μm.



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permeability and the alteration of tight junctions on p38α downregulation.

Downregulation of p38α Reduces Proliferation and Increases Apoptosis in Colon Tumor Cells

To investigate the molecular basis for the reduced tumorigenesis in p38α-$\Delta^{IEC\text{-}ERT2}$ mice, we analyzed the levels of cell proliferation and apoptosis. We found significantly reduced cell proliferation but increased apoptosis in the tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice compared to WT mice (Figure 7A). In agreement with the known importance of the cytokines IL-6 and IL-11 and the chemokines CXCL-1 and CXCL-2 for colon tumorigenesis (Grivennikov et al., 2009; Jamieson et al., 2012; Katoh et al., 2013; Putoczki et al., 2013), we found that IL-6, IL-11, CXCL-1, and CXCL-2 were all significantly downregulated in the colon tumors from p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure 7B). Both CXCL-1 and CXCL-2 attract cells expressing the CXCR2 receptor, mostly neutrophils and myeloid-derived suppressor cells that are positive for myeloperoxidase (MPO) staining and contribute to inflammation-driven tumorigenesis (Jamieson et al., 2012; Katoh et al., 2013). Reduced expression of CXCL-1 and CXCL-2 correlated with fewer MPO^+ cells in p38α-deficient tumors (Figure S6K). However, macrophage infiltration was not affected by downregulation of p38α in tumor cells (Figure 7C). We also found that p38α downregulation in colon tumors resulted in enhanced levels of phosphorylated JNK and reduced levels of phosphorylated STAT3, whereas the prosurvival pathways ERK1/2 and Akt were not affected (Figure 7D). Moreover, expression of the anti-apoptotic protein Mcl-1 was reduced in the p38α downregulated colon tumors, which may contribute to the enhanced apoptosis observed in p38α-$\Delta^{IEC\text{-}ERT2}$ mice (Figure 7E). These results support an important role for p38α in colon tumor maintenance by promoting proliferation and inhibiting apoptosis of the tumor cells.

Chemical Inhibition of p38α Reduces Colon Tumor Burden in Mice

The aforementioned findings were confirmed using PH797804, a chemical compound that potently inhibits p38α and is being used in clinical trials for inflammatory diseases (Goldstein et al., 2010). Colon tumors were induced using the AOM/DSS protocol, and then mice were separated in two groups that received either PH797804 or vehicle for 12 days (Figure 8A). We found that mice treated with the p38α inhibitor had a significantly reduced colon tumor load, reflecting both fewer tumors and smaller sizes compared to vehicle-treated mice (Figure 8B). IHC analysis confirmed the inhibition of colon tumorigenesis by administration of PH797804, which also reduced p38 MAPK signaling in the colon tumors and in adjacent normal epithelia (Figures 8C and 8D). These results extend our genetic analysis, indicating a protumorigenic role for p38α signaling in colon tumor cells. Moreover, incubation of Caco-2 and SW-620 human colon cancer cell lines with three different p38 MAPK chemical inhibitors resulted in enhanced apoptosis (Figure 8E), which correlated with increased levels of phosphorylated JNK (Figure 8E), as observed on p38α downregulation in mouse colon tumors.

Collectively, our results indicate that p38α signaling plays a dual role during colorectal tumorigenesis, suppressing the initial stages that lead to IEC transformation but contributing to tumor maintenance (Figure 8F).

DISCUSSION

In this study, we show that p38α suppresses colitis-associated colon tumor initiation by regulating the epithelial barrier function, which protects against epithelial damage and inflammation. However, once colon tumors are formed, p38α facilitates tumorigenesis by promoting proliferation and inhibiting apoptosis of the transformed epithelial cells.

In agreement with previous evidence for the tumor suppressor role of p38α in mouse models of lung and liver cancer (Hui et al., 2007; Ventura et al., 2007), we show that p38α downregulation in IEC enhances CAC. The AOM/DSS protocol is highly dependent on DSS, which induces epithelial apoptosis and inflammation. For example, downregulation of NEMO (NF-κB essential modulator) or STAT3 in IEC results in chronic colitis due to excessive IEC apoptosis (Bollrath et al., 2009; Nenci et al., 2007). Similarly, we show that p38α-deficient IEC undergo extensive apoptosis on DSS exposure, which correlates with the accumulation of the proapoptotic protein Bak. It has been proposed, in a different context, that negative regulation of Bak expression by p38α attenuates ionizing radiation-induced cell death (Kim et al., 2008b). The increased apoptosis observed in DSS-treated p38α-Δ^{IEC} mice extends a recent report showing more epithelial damage in p38α-deficient than in WT mice treated with DSS (Otsuka et al., 2010). In addition, we have found enhanced DSS-induced inflammatory cell infiltration in p38α-Δ^{IEC} mice. Different DSS treatment conditions might account for the discrepancies.

DSS-treated p38α-Δ^{IEC} mice produce increased levels of IL-6, a critical cytokine for colorectal tumorigenesis that regulates IEC

Figure 5. Downregulation of p38α in IEC Affects Intestinal Homeostasis and Paracellular Permeability

(A) Quantification of proliferating $Ki67^+$ cells, PAS^+ goblet cells, and $ChgA^+$ enteroendocrine cells. Data are means ± SEM (n = 4). *p < 0.05.

(B) Intestinal permeability was measured by determining the concentration of FITC-dextran in blood serum. Data are means ± SEM (n = 4). *p < 0.05.

(C) Representative electron microscope images showing tight junctions in colon epithelia. Scale bars, 50 nm. At least 25 tight junctions were analyzed. Histogram shows the quantification of the number of kissing points per tight junction. Data are means ± SEM. *p < 0.05.

(D) Colon lysates were analyzed by western blotting (one mouse per lane) with the indicated antibodies.

(E) Mice were treated with PBS or probiotic mixture VSL#3 for 1 week, and intestinal permeability was determined as in (B). Data are means ± SEM (n ≥ 3). *p < 0.05.

(F) Mice were treated as in (E) and then with 3% DSS for 3 days. $TUNEL^+$ apoptotic cells were quantified. Data are means ± SEM (n = 3). *p < 0.05.

(G) Mice were treated as in (E) and then with 2% DSS for 5 days. Body weights were recorded before starting the DSS treatment and at day 6. Data are means ± SEM (n ≥ 4). *p < 0.05.

(H) Mice were treated as in (G) and killed at day 6. Colon sections were stained with H&E and histologically analyzed. Scale bars, 50 μm. The histogram shows quantification of the epithelial damage. Data are means ± SEM (n ≥ 3). *p < 0.05.

See also Figure S4.

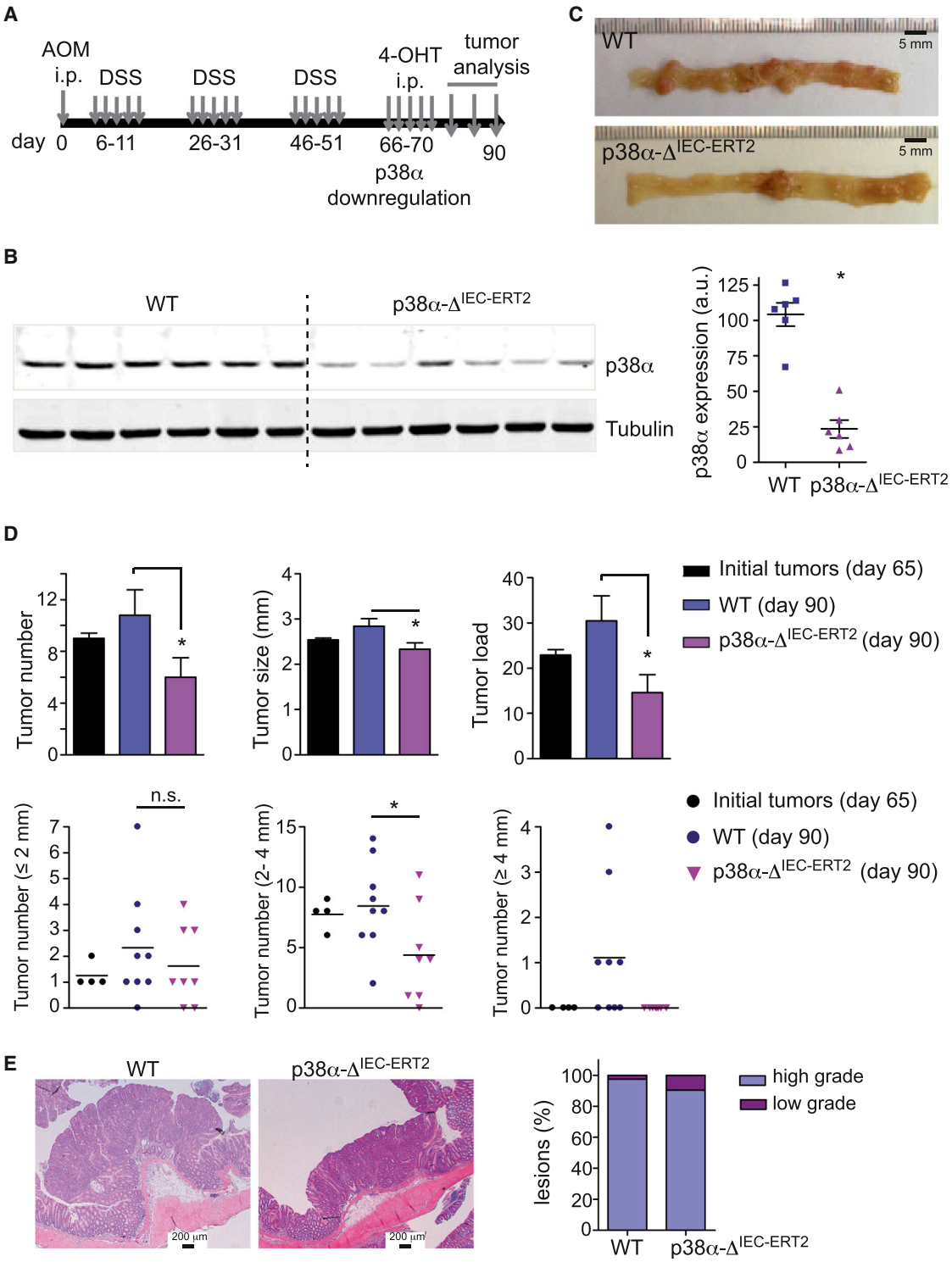

Figure 6. Downregulation of p38α in Colon Tumor Cells Reduces Tumor Burden

(A) Schematic representation of the protocol used to downregulate p38α in AOM/DSS-induced colon tumors.

(B) Colon tumors were analyzed by western blotting (one mouse per lane). Quantification is shown in the right panel. Data represent means ± SEM (n = 6). *$p < 0.05$.

(C) Representative images of colon tumors.

(D) Average tumor number, size, load, and tumor size distribution in initial tumors (day 65) and 20 days after the last 4-OHT injection (day 90). Data represent means ± SEM (n = 4 for initial tumors and n = 8 for WT and p38α-$\Delta^{IEC-ERT2}$). *$p < 0.05$.

(E) Colon tumor sections were stained with H&E and classified into low or high grade based on histological analysis (n = 8).

See also Figure S5.

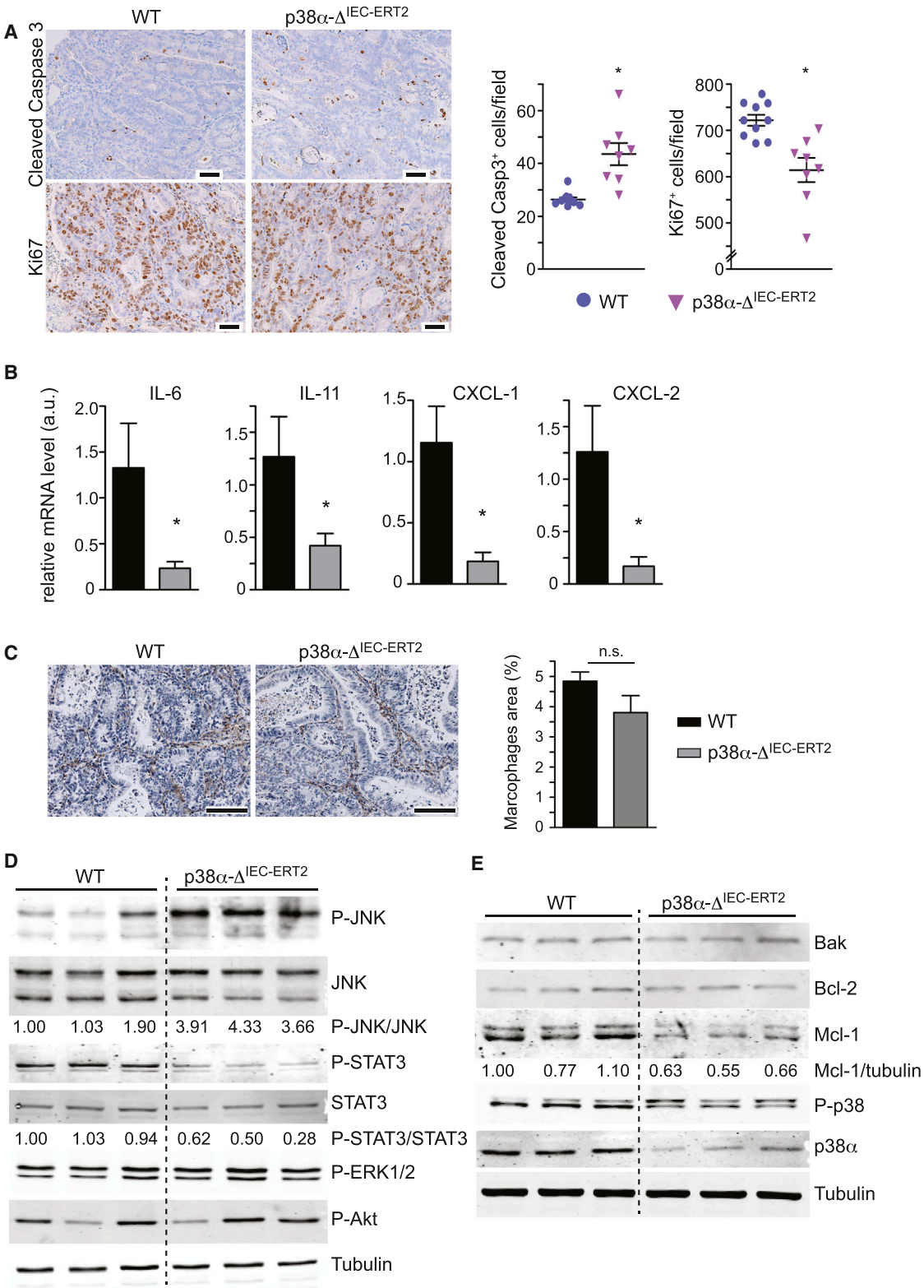

Figure 7. Downregulation of p38α Reduces Proliferation and Increases Apoptosis in Colon Tumor Cells

(A) Colon tumor sections were stained with the indicated antibodies. Scale bars, 50 μm. Quantifications are shown in the right panels. Data represent means ± SEM (n = 8). *p < 0.05.

(B) Relative mRNA expression levels for the indicated genes were determined by qRT-PCR. Data are means ± SEM (n = 4). *p < 0.05.

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survival and proliferation and whose protumorigenic functions relay on the activation of the transcription factor STAT3 (Becker et al., 2004; Bollrath et al., 2009; Grivennikov et al., 2009). Sustained activation of the NF-κB pathway also contributes to tumor development by inducing expression of inflammatory mediators and growth factors (Greten et al., 2004). Consistent with these results, the STAT3 and NF-κB pathways are both upregulated in p38α-Δ^{IEC} mice after DSS-induced colitis. Enhanced damage and the inflammation-associated release of cytokines probably contribute to the hyperproliferation of IEC in p38α-Δ^{IEC} mice on repetitive DSS-induced epithelial injury.

Our results demonstrate the importance of p38α signaling in intestinal homeostasis and integrity of the colon epithelia. Of particular importance is the reduced number of mucus-producing goblet cells, which we and others (Otsuka et al., 2010) have observed in p38α-Δ^{IEC} mice. Mucins produced by goblet cells form a protective mucous layer, which serves as a first barrier to pathogens or chemical injury (Rescigno, 2011). Accordingly, *Muc2* knockout mice spontaneously develop colitis and intestinal tumors, but with a very low incidence (1.5 tumors per mouse at 1 year of age) (Van der Sluis et al., 2006; Velcich et al., 2002). However, *Muc2*$^{+/-}$ mice do not develop spontaneous colitis but are more sensitive to DSS-induced colitis (Van der Sluis et al., 2006). Similarly, the decreased expression of MUC2 in p38α-Δ^{IEC} mice is not sufficient to induce spontaneous colitis or tumors but is likely to facilitate DSS-induced colitis and colon tumor formation as in *Muc2*$^{+/-}$ mice.

The importance of intestinal epithelial integrity is emphasized by the altered paracellular permeability and tight junction functions observed in IBD patients (Schmitz et al., 1999; Westbrook et al., 2010). Moreover, it has been recently reported that colon tumors have defective epithelial barrier function, which has been associated with upregulation of IL-23 and IL-17 (Grivennikov et al., 2012). Mice lacking the tight junction protein JAM-A also show increased intestinal permeability and are more susceptible to DSS-induced colitis (Laukoetter et al., 2007). Downregulation of p38α in IEC affects epithelial barrier function, which does not correlate with IL-23 and IL-17 upregulation but with reduced expression of ZO-1, an important regulator of tight junction assembly. The probiotic mix VSL#3 can improve defective barrier function in a model of acute DSS-induced colitis (Mennigen et al., 2009). Experiments with VSL#3 support an important role for p38α in the regulation of intestinal barrier integrity, which protects against colitis-induced epithelial damage and the initiation of colon tumorigenesis. However, probiotics can also modify the gut microbiota, which might provide further beneficial effects by restoration of microbial communities and suppression of pathogens and immunomodulation (Preidis and Versalovic, 2009).

In contrast to the in vivo tumor suppressor function of p38α during the onset of several types of cancer, p38α might facilitate cancer cell proliferation and survival in established human cell lines and in some mouse models (reviewed by Wagner and Nebreda, 2009). These studies are mainly based on the use of SB203580 and SB202190, two chemical inhibitors that are known to have off-target effects (Fabian et al., 2005). For example, the induction of autophagy by SB202190 in colorectal cancer cells (Chiacchiera et al., 2009; Comes et al., 2007) has been ascribed to nonspecific effects rather than to the inhibition of p38α signaling (Menon et al., 2011). Here, we provide genetic and pharmacological evidence supporting the implication of p38α in colon tumor maintenance.

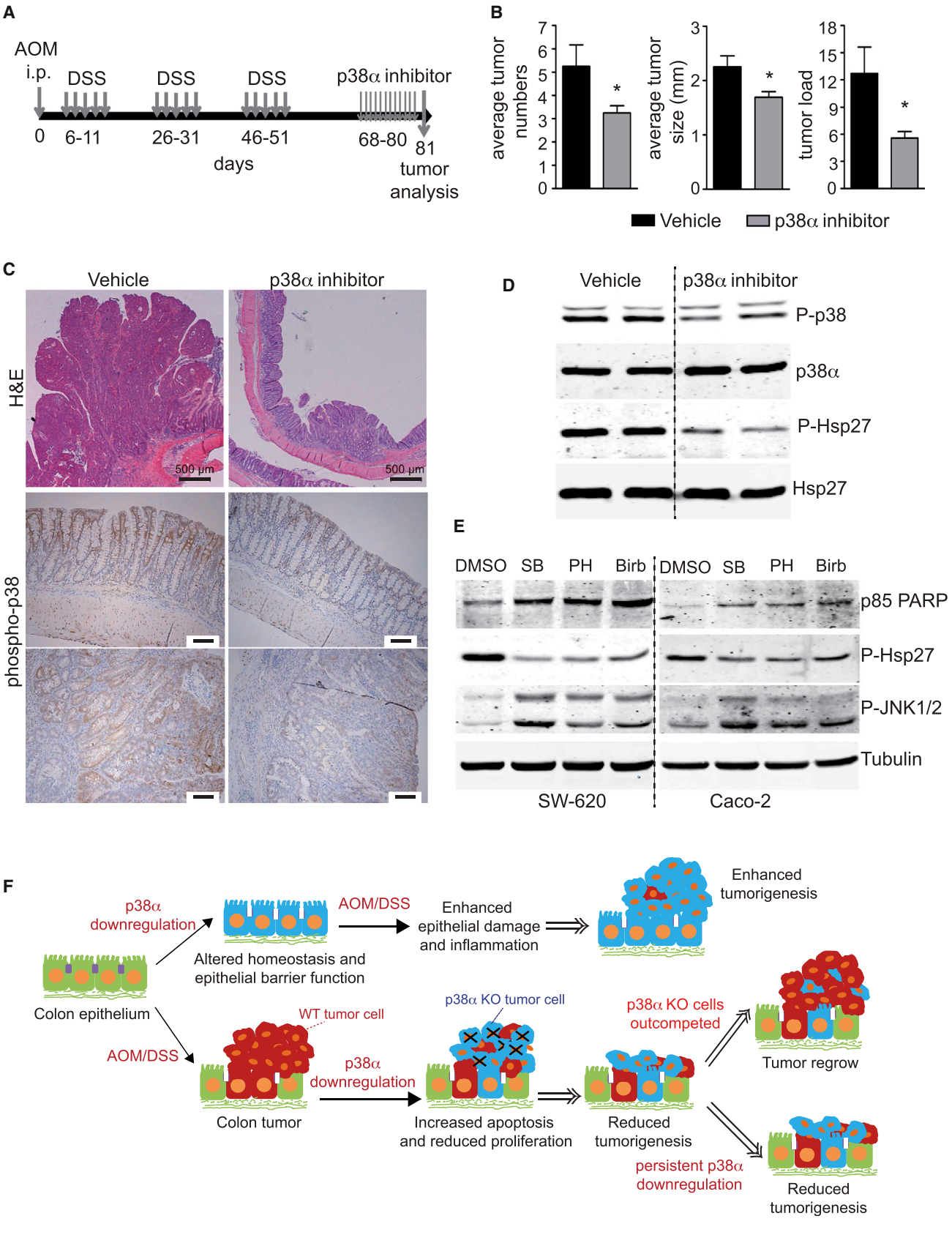
The defective barrier function of colon tumors results in increased permeability, which contributes to tumorigenesis by facilitating an enhanced inflammatory response and can also enable invasiveness by reducing adhesion of tumor epithelial cells (Grivennikov et al., 2012; Schwitalla et al., 2013). Given the role of p38α in normal epithelial barrier function, p38α downregulation could further enhance tumor permeability, which should promote tumorigenesis. However, our results show that p38α does not regulate tumor permeability, which is severely affected in colon tumors compared with normal intestine. Moreover, the downregulation of p38α reduces tumor burden without increasing tumor invasiveness, suggesting that p38α is unlikely to regulate the barrier function in tumors.

Previous studies showed that STAT3 is an important regulator of colon tumor cell survival and proliferation (Bollrath et al., 2009; Grivennikov et al., 2009), whose activation is strongly associated with expression of IL-6 and IL-11. Inhibition of STAT3, IL-6, or IL-11 greatly reduces tumor burden in inflammation-associated colon cancer (Grivennikov et al., 2009; Putoczki et al., 2013). The p38α pathway can control the production of proinflammatory cytokines (Cuenda and Rousseau, 2007). We have shown that expression of IL-6 and IL-11 is reduced in the tumors on p38α downregulation, which also correlates with reduced phosphorylation of STAT3. Autocrine mechanisms, such as the production of the aforementioned cytokines, could account for the observed link between p38α activity and STAT3 phosphorylation, although we cannot rule out that p38α might also regulate intracellular signaling pathways leading to STAT3 phosphorylation in tumor cells.

Inactivation of p38α in colon tumors induces significant apoptosis, which correlates with reduced expression of the antiapoptotic protein Mcl-1 and increased phosphorylation of JNK. It is interesting that p38α can negatively regulate JNK signaling by several mechanisms (Wagner and Nebreda, 2009), and sustained activation of JNK has been linked to the induction of apoptosis (Ventura et al., 2006). Moreover, JNK has been reported to mediate phosphorylation and degradation of Mcl-1 (Morel et al., 2009). Altogether, it seems likely that activation of JNK signaling together with reduced expression of Mcl-1 contribute to the increased apoptosis observed in colon tumors on p38α downregulation.

The reappearance of p38α-expressing tumors at 45 days after 4-OHT administration suggests that Villin-CreERT2 induction does not delete p38α in all transformed epithelial cells and that the WT escapers eventually repopulate the tumors (Figure 8F). Thus p38α-deficient tumor cells are at a selective disadvantage and are outcompeted by WT tumor cells, which is consistent with the increased apoptosis and reduced proliferation observed in

(C) Colon tumor sections were stained with F4/80 to detect macrophages. Scale bars, 100 μm. Quantifications are shown in the right panels. Data represent means ± SEM (n = 3).
(D and E) Colon tumor lysates were analyzed by western blotting (one mouse per lane) with the indicated antibodies.
See also Figure S6.



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tumors on p38α downregulation. It is possible that the inducible Villin-CreERT2 might not be so effective in cancer intestinal stem cells. However, Villin-CreERT2 has been shown to be active in all IEC, including putative progenitor cells in the crypts (el Marjou et al., 2004), as well as during APC (adenomatous polyposis coli)-induced polyp formation (Sonoshita et al., 2011). Moreover, global gene expression analysis has shown that normal intestinal stem cells are very similar to the cancer stem cells (Merlos-Suárez et al., 2011). It is, therefore, likely that Villin-CreERT2 works in normal intestinal stem cells as well as in all compartments of the intestinal epithelia during cell transformation and adenoma progression. In any case, it seems that p38α promotes viability of the majority of colon tumor cells, based on the substantially impaired colon tumor growth observed on downregulation or inhibition of p38α, and the observation that colon tumors do not regrow when p38α downregulation is maintained.

Our results also provide an example of a nononcogenic addictive pathway that is important for tumor maintenance (Luo et al., 2009). The requirement of p38α for colon cancer cell survival and proliferation does not imply that the pathway should be necessarily upregulated in colon tumor cells. Nevertheless, enhanced levels of phosphorylated p38 MAPK have been detected in human colon tumor samples, both in cancer cells and in stromal cells (Hardwick et al., 2001; Paillas et al., 2011).

In summary, we describe a dual role of p38α signaling in colon tumorigenesis, suppressing inflammation-associated colon tumor initiation but contributing to tumor maintenance (Figure 8F). It has been reported that the p38α pathway can play different roles during skin tumorigenesis in epithelial or endothelial cells (Yoshizuka et al., 2012). Our in vivo experiments indicate that p38α functions as tumor suppressor or promoter in normal and transformed IEC, respectively. It remains to be determined how p38α signaling in other cell types contributes to colorectal tumorigenesis.

EXPERIMENTAL PROCEDURES

Mice

p38α-Δ^{IEC} mice and p38α-$\Delta^{IEC\text{-}ERT2}$ mice were generated by crossing p38α$^{lox/lox}$ mice (Ventura et al., 2007) with Villin-Cre and Villin-CreERT2 mice (el Marjou et al., 2004), respectively. The Villin-Cre mice were mostly in C57BL/6 background, while Villin-CreERT2 mice were of mixed C57BL/6-129v background. Littermate controls were used in all experiments. Mice were housed according to national and European Union regulations, and protocols were approved by the animal care and use committee of the Barcelona Science Park.

Induction of Colitis and CAC

To induce colorectal tumors, we used a combination of the carcinogen AOM with repeated administration of DSS in the drinking water, which causes colitis (Neufert et al., 2007). Mice (8–10 weeks old) were injected intraperitoneally with a single dose of AOM (10 mg/kg; Sigma, #A2853). After 5 days, 2% DSS (molecular weight, 36–50 kDa; MP Biomedicals, #160110) was given in the drinking water for 5 days, followed by 14 days of regular drinking water. The DSS treatment was repeated for two additional cycles, and mice were sacrificed 100 days after the AOM injection, except when indicated otherwise. For short-term colitis and inflammation studies, mice were given 2% DSS for 5 days and sacrificed at the indicated time points. Body weights were recorded during DSS treatment. Colons were removed from mice, flushed with cold PBS, opened longitudinally, fixed as "swiss-rolls" in 10% formalin solution (Sigma, #HT-501128) at room temperature overnight, and paraffin embedded. Before fixing the colons, size measurements were performed using a digital caliper in a blinded fashion.

Histopathological Analysis

Paraffin-embedded colon sections were stained with hematoxylin and eosin (H&E) and analyzed by pathologists in blinded fashion for tumor grades, epithelial damage, and inflammation using the scoring systems described in the Supplemental Information.

IHC

For IHC, colon sections were stained with the antibodies indicated in the Supplemental Information. Signals were visualized with 3,3-diaminobenzidine, using hematoxylin as a counterstain. PAS reagent was used to detect goblet cells.

TUNEL Assay

Apoptosis was detected in paraffin-embedded colon samples using the Fluorescein In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Images were taken with a Nikon E800 upright microscope using appropriate fluorescence filters.

Analysis of Intestinal Permeability in Mice

To determine in vivo intestinal permeability, mice were starved overnight, and then FITC-dextran (Sigma #FD4) was administered by oral gavage (44 mg/100 g body weight). After 4 hr, mice were anesthetized, blood was collected by cardiac puncture, and mice were sacrificed. Serum was separated from whole blood using BD Microtainer SST Tubes (BD #365968), diluted with an equal volume of PBS (pH 7.4), and 100 μl of diluted serum was added to a 96-well microplate. The concentration of FITC in serum was determined by spectrophotofluorometry (BioTek), with an excitation of 485 nm and an emission wavelength of 528 nm, using serially diluted FITC-dextran as standard. Tumor permeability was calculated as described in the Supplemental Information.

Probiotic Treatment

Probiotic mixture VSL#3 (15 mg, Grifols) was dissolved in PBS (200 μl) and administered daily by oral gavage. Control mice were administered PBS.

Statistical Methods

Data are presented as means ± SEM. Statistical significance was determined by Student's t test using GraphPad Prism 4 software. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.02.019.

Figure 8. Chemical Inhibition of p38α Reduces Colon Tumor Burden

(A) Schematic representation of the protocol used to induce colorectal tumors in C57BL/6 mice and for administration of the p38α inhibitor PH797804.

(B) Average tumor number, size, and load at the end of the AOM/DSS protocol. Data are means ± SEM (n = 7). *p < 0.05.

(C) Representative images of colon sections stained with H&E or the phospho-p38 MAPK antibody at the end of the AOM/DSS protocol. Scale bars, 100 μm for phospho-p38.

(D) Colon lysates were analyzed by western blotting (one mouse per lane) with the indicated antibodies.

(E) Human colon cancer cell lines were treated with the p38 MAPK inhibitors SB203580 (SB, 10 μM), PH797804 (PH, 1 μM) or Birb0796 (Birb, 200 nM) for 4 days, and cell lysates were then analyzed by western blotting with the indicated antibodies.

(F) In normal colon epithelial cells, p38α maintains intestinal homeostasis and barrier function to suppress colitis-associated tumor initiation. On the other hand, p38α contributes to colon tumor development by supporting proliferation and inhibiting apoptosis of transformed epithelial cells. Tumor cells deficient in p38α appear to be at a selective disadvantage compared with WT cells.

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